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Review

miRNA Multiplayers in glioma. From bench to bedside

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Glioblastoma multiforme (GBM) is the most common type of malignant gliomas, characterized by genetic instability, intratumoral histopathological variability and unpredictable clinical behavior. Disappointing results in the treatment of gliomas with surgery, radiation and chemotherapy have fuelled a search for a new therapeutic targets and treatment modalities. A novel small noncoding RNA molecules, microRNAs (miRNAs), appear to represent one of the most attractive target molecules contributing to the pathogenesis of various types of tumors. They play crucial roles in tumorigenesis, angiogenesis, invasion and apoptosis. Some miRNAs are also associated with clinical outcome and chemo- and radiotherapy resistance. Moreover, miRNA have the potential to affect the responses to molecular-targeted therapies and they also might be associated with cancer stem cell properties, affecting tumor maintenance and progression. The expression profiles of miRNAs are also useful for subclassification of GBM, what underscores the heterogeneity of diseases that all share the same WHO histopathological grade. Importantly, molecular subtypes of GBM appear to correlate with clinical phenotypes, tumor characteristic and treatment outcomes. miRNAs are then biological markers with possible diagnostic and prognostic potential. They could also serve as one of the promising treatment targets in human glioblastoma.

Key words: cancer, glioblastoma multiforme (GBM), miRNA, anti-cancer therapy, diagnosis, prognosis

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miRNA — THE NEW PLAYERS IN CANCER

The central dogma of molecular biology states that the transfer of genetic information within cells goes sequentially from DNA to RNA to proteins, whose coding sequences comprise about 1.5–2% of the human genome (Li & Xie, 2011; Esteller, 2011). Although genetic and epigenetic aberrations that occur in components of central dogma pathway clearly elicit disease development in humans, recent findings also point to a prominent role for non-protein-coding regions of the genome in regulation of cell and tissue homeostasis, as well as in contributing to the formation of human tumors. The functional relevance of these regions is particularly evident for a class of small non-coding RNAs (ncRNAs) — microRNAs (miRNAs) (Esteller, 2011). These types of ncRNAs, together with other ncRNAs, such as PIWI-interacting RNA (piRNA), small nucleolar RNA (snoRNA), the large intragenic ncRNA (lincRNA), the ultraconserved transcribed regions (T-UCR) and, overall, the heterogeneous group of long non-coding RNAs (lncRNAs), might also contribute to the development of many different regulatory processes and human disorders (Esteller, 2011; Ling *et al*., 2013). However, the best characterized and most extensively studied are miRNAs, which play essential functions during embryogenesis and tissue development, cell proliferation, differentiation and survival (Esteller, 2011; Ling *et al*., 2013). miRNAs are small (17–27 nt) ncRNAs that govern gene expression in a post-transcriptional manner by binding to the target mRNAs, thereby repressing their translation or inducing their degradation (Ambros, 2004). Primary miRNAs (pri-miRNA) are transcribed in the nucleus by polymerase II. pri-miRNA is then cleaved into a short about 70 nucleotides hairpin precursor (pre-miRNA) by Drosha nucleases. This nuclear processing is followed by the transport of pre-miRNA from the nucleus into the cytoplasm *via* exportin-5 and then by the further processing for mature miRNA by Dicer complexes. Mature miRNA is incorporated into an effector complex known as an RNA-induced silencing complex (RISC), which binds to mRNA and can affect the translation and stability of mRNA (Filipowicz *et al*., 2008). Expression of the target mRNA is regulated, either by mRNA cleavage or by translational repression, depending on the complementarity of "seed" sequences (Filipowicz *et al*., 2008). Many reports have revealed that miRNAs play crucial roles in tumorigenesis, angiogenesis, invasion, and apoptosis in various types of tumor (Ambros, 2004; Bartel, 2004).

Although the role of miRNAs in cancer pathogenesis is evident, however it is still not known whether the deregulation of miRNAs is a reason or a consequence of cancer transformation (Nicoloso *et al*., 2009; Ventura & Jacks, 2009). miRNAs are frequently located at fragile genome sites or regions that are very often amplified or

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Abbreviations: 2′F, 2′-Fluoro; 2′MOE, 2′O-methyoxethyl; 3′ UTR, 3′ untranslated region; AVV, adenovirus associated vectors; AMOS, antisense miRNA nucleotides; BBB, blood- brain barrier; CDK 4/6, cyclin-dependent kinase 4/6; CED, convection-enhanced delivery; CSC, cancer stem cells; CSF, cerebrospinal fluid; dsRNA, double-stranded RNA; EGFR, epidermal growth factor receptor; GBM, glioblastoma multiforme; IDH 1, isocitrate dehydrogenase 1; JNK, C-Jun N-terminal kinase**;** lincRNA, large intergenic RNA; LOH, loss of heterozygosity; MDM1, mouse double minute 1; MGMT, 0-6-methylguanine-DNA methyltransferase; miRNA, micro RNA; ncRNA, non-coding RNA; NF1, neurofibromin 1; PDGF, plateled-derived growth factor; PI3K, phosphoinositide kinase; piRNA, PIWI- interacting RNA; pri-miRNA, primary miRNA; PTEN, phosphatase and tensin homolog; RB1, retinoblastoma 1; RECK, reversion-inducing-cysteine-rich protein; RNAi, RNA interference; RTK, receptor tyrosine kinase; snoRNA, small nucleolar RNA; STAT3, signal transducer and activator of transcription; TCGA, The Cancer Genome Atlas; TMZ, temozolomide, TIMP1, metallopeptidase inhibitor 1; TN-C, tenascin C; TP53, tumor protein 53; TRAIL, TNF-related apoptosis-inducing ligand; T-UCR, ultraconserved regions; UPAR-urokinase receptor; WHO, World Health Organization; VEGFR, vascular endothelial growth factor receptor

deleted in human cancers (Calin *et al*., 2004). Emerging evidence indicates that miRNAs function as molecular rheostats that serve in fine-tuning cell signalling pathways by modulating the expression of large numbers of genes and consequently, impacting the flux through essential regulatory nodes of vast signaling networks. These functional characteristic enhances the belief that targeting and manipulating either the expression or activity of miRNAs may provide novel inroads to treatment of human cancers.

THE HALLMARKS OF CANCER

The hallmarks of cancer comprise the six biological capabilities acquired during the multistep development of human tumors. These features of cancer all together constitute basis that provides a framework for understanding the remarkable diversity of neoplastic diseases. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Reprogramming energy metabolism and evading immune destruction are additional cancer features in contrary to the normal cell (Hanahan & Weinberg, 2011).

A recent discovery of hundreds of distinct regulatory miRNAs has already led to dramatic changes in our understanding of the genetic control mechanisms. By now miRNAs have been implicated in various tumor phenotypes, but theirs function in cells and altered expression in different forms of cancers still remain poorly understood (Garzon *et al*., 2010).

GLIOMA CHARACTERISTICS

Gliomas are a heterogenous group of tumors classified by the World Health Organization (WHO) into pilocytic astrocytomas (WHO I) with slow growth and rarely undergoing malignant transformation and three groups of diffusely infiltrative astrocytomas comprising diffuse astrocytomas (WHO II), anaplastic astrocytomas (WHO III) and glioblastoma (WHO IV) (Louis *et al*., 2007).

As described by the WHO classification, malignant diffuse gliomas are comprised of astrocytic, oligodendroglial and mixed oligoastrocytic neoplasm based solely on morphology and are further subdivided by tumor grade based on additional histologic features present in the tumor (Louis *et al*., 2007).

Glioblastoma multiforme (GBM), as indicated by the word "multiforme", displays a highly heterogeneous composition of cells and exhibit phenotypic heterogeneity because it is composed of cells that express markers of both undifferentiated and differentiated cells. The histologically defined groups of astrocytic, oligodendroglia and oligoastrocytic (mixed) gliomas of WHO grades II and III remain a major challenge in various ways: (1) there is poor interobserver agreement when diagnoses and grading are made by histological criteria alone, (2) the clinical course is highly variable, and (3) the clinical management remains poorly standardized (van den Bent, 2010).

Nuclear atypia and mitotic activity are required criteria for grade III lesions, and the presence of necrosis or microvascular proliferation is required for GBMs (Louis *et al*., 2007). GBM is associated with such histopathological features as cellular polymorphism, atypia, substantial mitotic activity (with 3–5 fold higher proliferative rates than grade III anaplastic astrocytoma), vascular thrombosis and the most essential in terms of diagnosis — microvascular proliferation and necrosis. Other prominent features of GBM are regional heterogeneity, highly invasive growth and diffuse infiltration of the surrounding brain (Kleihues & Ohgaki, 1999; Kleihues *et al*., 2002, Ohgaki & Kleihues, 2007; 2009).

GBM is moreover characterized by genetic instability and complex alterations in chromosome structure and copy number. The most significantly somatically mutated genes are: *TP53*, *PTEN* (*Phosphatase and Tensin Homology*), *NF1* (*neurofibromatosis-1*), *EGFR (Epidermal growth factor receptor*), *RB1* (*retinoblastoma-1*) gene and *PIK3R1* (Dunn *et al*., 2012). Both primary and secondary glioblastomas arise from precursor cells that may be distinct. Primary GBM arise *de novo* and exhibit p53and Rb pathway dysfunction as well as RTK/Ras/PI3K signaling dysregulation, leading to the tumors that arise in older patients with a worse prognosis, likely owing to the predominant wild type *IDH1* genotype. In contrast, secondary GBMs are preceded by lower — grade II lesions, which progress either through grade III lesions or directly to GBM. These tumors occur in younger patients and are dominated by a mutant IDH1 genotype that confers a better prognosis and is associated with a more restricted frontal lobe location (Louis *et al*., 2007; Dunn *et al*., 2012).

GBM have been categorized into primary and secondary tumors, on the basis of clinical presentation. Primary GBM arises *de novo* without evidence of prior glioma precursor, whereas secondary glioblastoma progress from previously diagnosed lower-grade brain tumors. Primary GBM account for the majority of all GBM and are more frequent in older patients, while secondary GBM is quite rare and tends to occur in patients below the age of 45 years (Kleihues & Ohgaki, 1999; Kleihues *et al*., 2002). Although primary GBM is indistinguishable from secondary GBM by histology, these two types of tumor exhibit distinct genetic alterations. In *de novo* (primary) GBM, EGFR gene amplification is often combined with gene rearrangements on chromosome 7p that lead to constitutively active, truncated receptor (EGFR variant III — EGFRvIII) (Halatsch *et al*., 2006). EGFR or EGFRvIII overexpression is usually associated with deletion of *INK4a/p14ARF* gene locus and loss of wild-type *p53* expression as well as PTEN. The hallmarks of secondary GBM are *p53* mutations and overexpression of platelet-derived growth factor (PDGF) and its receptor (PDGFR) (Halatsch *et al*., 2006). The inactivation of other suppressor genes as *p16, RB, PTEN* and activation of such oncogenes as the human homolog of the Mouse Double Minute 2 (*MDM2*) and Cyclin-Dependent Kinases 4/6 (*CDK4/6*) are also frequently observed during progression of low-grade gliomas to GBM (Halatsch *et al*., 2006; Reardon *et al*., 2006). The differences between primary and secondary GBMs are also observed at the chromosomal level, as showed with karyotyping, chromosomal painting or comparative genomic hybridization (CGH) techniques. The most common chromosomal alterations in primary GBM are amplifications and gains of 7p, 12q13-q21, chromosome 19 and the regions of chromosomal losses are: 10q, 9q, 13q, 22q (Koschny *et al*., 2002; Roerig *et al*., 2005). Apart from differences between the genetic lesions found in secondary and primary GBMs, a multiplicity of genetic aberrations are observed across individuals with the same tumor type and sometimes within the same tumor of a given individual.

Epigenetic modifications of cytosines (5-methylocytosine formation) in DNA, also effect gene function in gliomas. For example, the promoter of O-6-methylguanine-

DNA methyltransferase (MGMT), an enzyme involved in DNA repair, is frequently silenced by hypermethylation in many cancers including gliomas (Esteller & Herman, 2004).

GBM is one of the most common tumors in adults and constitutes 25% of all malignant nervous system tumors (Loius, 2007). Median overall survival remains around 14.6 months, and the 5-year survival rate is only 9.8% at present (Stupp *et al*., 2009).

Although GBM is diagnosed on the basis of their histopathological morphological features, it has been demonstrated that molecular heterogeneity among glioblastomas is prominent, and pathological diagnosis cannot always predict behaviour of the tumor. Although recent medical treatment strategies have been progressing toward individualized therapy and many targeted drugs have been investigated, the identification of molecular biomarkers in GBM will be still of considerable therapeutic importance.

GBM THERAPY

Standard treatment

Fluorescence image-guided tumor removal with intraoperative neuro-functional monitoring increases the possibility of maximum tumor resection without neurological deficit, which prolongs patient survival (Stummer *et al*., 2006; Sanai *et al*., 2009). Present radiotherapy modalities such as intensity-modulated radiation therapy and heavy charged particle therapy have been investigated to improve the treatment efficacy of conventional radiation therapy (Sultanem *et al*., 2004; Mizoe *et al*., 2007). Moreover, temozolomide (TMZ), an oral alkylating chemotherapeutic agent, has been demonstrated to enhance patient survival (Reardon *et al*., 2006; Stupp *et al*., 2006). Additionally, molecular targeted drugs such as EGFR inhibitors and VEGFR antibodies have been used clinically (Wick *et al*., 2011). However, despite this technical advancement of therapeutic modalities, the treatment of patients with GBM has only improved minimally, with a median survival time (Stupp *et al*., 2005; 2009). However, all these efforts suggest that effective therapeutic targets still remain to be identified.

Detailed analysis of patients' brain tumor samples and glioma cell lines has identified numerous genes that are important for the regulation of signaling networks responsible for sustained cellular proliferation in malignant gliomas. The main oncogenetic signaling molecular pathways identified in human malignant gliomas include growth factors, phosphatidylinositol 3-kinase (PI3K)/AKT/PTEN/mammalian target of rapamycin (mTOR), Ras/Raf/mitogen-activated protein kinase (MAPK), and sonic hedgehog/PTCH. The first trials of targeted therapy of malignant gliomas have concerned inhibition of known genetic alterations like mentioned RAS/MAPK and PI3K/AKT/mTOR pathways and tyrosine kinase receptors (EGFR, PDGR, VEGFR) by small-molecule inhibitors (Reardon *et al*., 2006; Omuro *et al*., 2007). Several drugs have been tested, including EGFR tyrosine kinase inhibitors (gefitinib and erlotinib), mammalian target of rapamycin (mTOR) inhibitors (rapamycin, temsirolimus and everolimus), and VEGFR, protein kinase C-â (enzastaurin), and other angiogenesis pathways inhibitors (vatalanib, bevacizumab, and enzastaurin). Phase I data of such inhibitors like gefitinib or erlotinib showed that the drugs were well tolerated. However, phase II data suggested that, although some responses were obtained, the overall efficacy of such agents in unselected patients was minimal when compared with historical data (Reardon *et al*., 2006; Omuro *et al*., 2007). The next generation of trials is exploring the possibility of addressing multiple targets through the use of multitargeting single agents, combinations of single-targeting agents, and combination with cytotoxic chemotherapy and/or radiotherapy are in study.

Experimental RNAi therapy

Despite huge efforts leading to the GBM treatment and in the light of unsatisfactory results of first molecu-

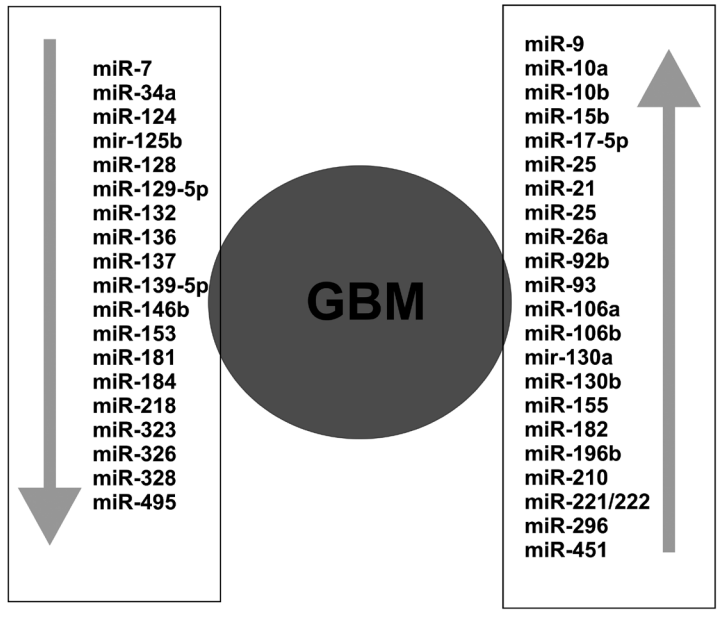

Figure 1. miRNAs consistently deregulated in glioblastoma.
The most common overexpressed miRNAs in GBM are shown with an "up" arrow and downregulated — with a "down" arrow (Ciafre *et al*., 2005; Piwecka *et al*., 2015).

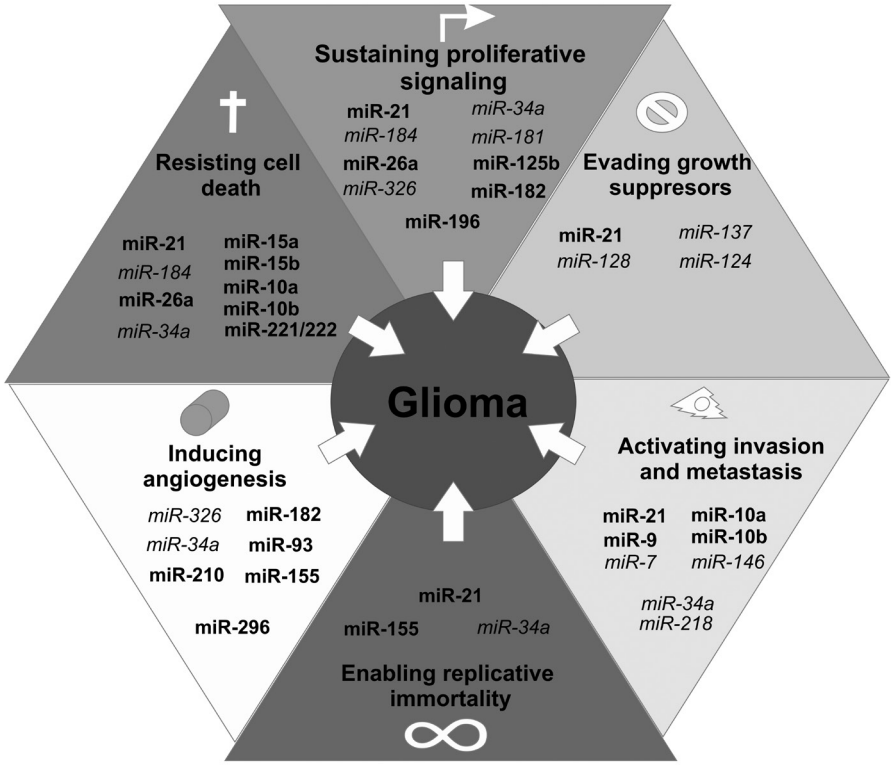

Figure 2. Aberrant miRNA expression affecting signalling GBM tumorigenesis pathways.
Representative miRNA are depicted as the oncomiRs (bold) or tumors supressors (italic) to affect the six main hallmarks of cancer (based on: Hanahan & Weinberg, 2011).

lar targeted therapies against RTKs that have not translated into significant changes in current clinical practice of malignant gliomas, the strong need arise for validating and implementing new molecular targets to GBM therapy.

Thus, we have proposed a few potential new protein targets for GBM treatments together with the specific and efficient molecular tools (e.g. catalytic nucleic acids: RNAi, ribozymes) (Pas *et al*., 2006; Piwecka *et al*., 2011). We designed and implemented the experimental therapy of patients suffering from malignant brain tumors based on application of double-stranded RNA (dsRNA) specific for tenascin-C (TN-C) mRNA. That therapeutic agent, called ATN-RNA, induces RNAi pathway to degradation of TN-C, the extracellular matrix (ECM) protein which is highly overexpressed in brain tumor tissue (Zukiel *et al*., 2007; Wyszko *et al*., 2008; Rolle *et al*., 2010). We have observed strong difference between *de novo* and recurrent tumors treated with ATN-RNA. The effect for ATN-RNA treated patients was the prolongation of the median survival for recurrent gliomas up to 100 weeks in contrary to standard therapy, whereas for patients with primary tumors — over 120 weeks (Zukiel *et al*., 2007; Wyszko *et al*., 2008; Rolle *et al*., 2010).

miRNAs IN GBM

One of the hallmarks of cancer are defects in the regulatory circuits that control normal cell proliferation and homeostasis. Previously, great efforts were focused on understanding the roles of protein-coding genes in cancer. At present, emerging studies implicate miRNAs as a novel class of non-coding tumor suppressor and oncogenes that play important roles in tumorigenesis.

By negatively regulating mRNA targets to either degradation or translational repression, they can act as both tumor supressors and oncogenes. miRNA has the capacity to regulate a large number of target mRNAs, often belonging to a single signalling pathway (Croce, 2009). As a new layer of gene-regulation mechanism, miRNA have diverse functions, including the influence on cellular differentiation, proliferation and apoptosis, as well as cancer initiation and progression (Chan *et al*., 2005). Importantly miRNAs play active roles in modulating of the all physiological processes in carcinomas being a fundamentals of basic hallmarks of cancer. They typically act on multiple pathways and programs to elicit disease development. miRNAs involved in cancer development and progression can be divided in following groups: oncomiRs (tumor promoting miRNAs), tumor suppressive miRNAs and metastamiRs (metastasis promoting miRNAs) (Garzon *et al*., 2010; Chou *et al*., 2013; Yates *et al*., 2013) (Fig. 2). Recently, the concept of "oncomiR addiction", similar to the phenomenon of "oncogenic addiction" has been proposed (Medina *et al*., 2010). That hypothesis emphasizes the apparent dependence of some cancers on one or few genes for maintenance the malignant phenotype and shows that certain tumors addict to specific miRNAs, e.g. miR-21 for GBM, what could facilitate study of the therapeutic application of miRNAs in human cancer.

Distinct miRNA expression profiles have been associated with GBM, and oncogenic roles have been suggested for miR-21, miR-10b and miR-26a whilst up-regulated levels of miR-296 is associated with angiogenesis (Conti

et al., 2009; Li *et al*., 2009; Sasayama *et al*., 2009; Wang and Olson 2009). Significantly downregulated miR-326, miR-128, miR-181 and miR-181b, miR-7, miR-137 and miR-124a act as tumor suppresors in GBM (Godlewski *et al*., 2008; Shi *et al*., 2008; Silber *et al*., 2008; Kefas *et al*., 2009; Webster *et al*., 2009; Zhang *et al*., 2009). Our recent comprehensive analysis, together with the previous data of the expression level of miRNAs in GBM, is given in Fig. 1.

It was shown that the best studied GBMs miRNAs: miR-21, miR-26a, miR-221/222, miR-7 and mir-34a target mainly components of EGFR/PTEN/Akt and p53/pRB pathways. miR-21 is overexpressed in approximately 70% of tumor samples, being considered one of the most common upregulated miRNAs in gliomas. Its knockdown in glioma cells led to increased apoptosis, reduced invasiveness and inhibition of tumor growth *in vivo* (Chan *et al*., 2005; Corsten *et al*., 2007; Gabriely *et al*., 2008). Specifically, inhibition of miR-21 has been shown to confer cell survival and also enhance sensitivity of GBM cells to a number of chemotherapeutic agents and TNF-related ligands (Chan *et al*., 2005; Corsten *et al*., 2007). The anti-apoptotic effect of miR-21 appears to be effected in part *via* regulation of cell cycle as miR-21 knockdown induces G0-G1 cell cycle arrest. At the molecular level, miR-21 has been shown to activate the EGFR/Akt cell survival pathways through direct targeting of PTEN (Ren *et al*., 2010; Zhou *et al*., 2010). In addition, miR-21 also confers survival and cell cycle arrest via p53-mediated and mitochondrial apoptotic pathways in part via direct regulation of HNRPK and Tap63, a p53 homologue (Papagiannakopoulos *et al*., 2008).

miR-26a, as another oncomiR, was identified as a target of gene amplification together with RB1 and PI3K/Akt pathway oncogenes, CDK4 and CENTG, in approximately 15% of 176 primary GBMs characterized in TCGA database (Kim *et al*., 2010). Interestingly, upregulation of miR-26a, which has been shown to directly target PTEN, occurs at greater frequency in primary tumors with PTEN LOH and monoallelic expression and its overexpression functionally subsitutes for PTEN loss *in vivo* (Brennan *et al*., 2009; Huse *et al*., 2009). In addition to PTEN, miR-26a directly regulates RB1 and MEKK2 and miR-26a augments CDK4 oncogenic effects in glioma cells *in vitro* and *in vivo*. These findings therefore suggest that the miR-26a/CDK4/CENTG oncogenic cluster seen in primary gliomas cooperatively regulate multiple targets to modulate the cell survival/proliferative and apoptotic functions mediated by the Akt, RB1 as well as the JNK pathways in malignant gliomas (Kim *et al*., 2010).

One of the most highly expressed miRNA in GBM (up to 208 fold higher than in normal brain) is miR-10b (Silber *et al*., 2008; Huse *et al*., 2009). Its high expression correlates with increased grade and invasive phenotypes in gliomas (Sasayama *et al*., 2009). The significant correlation of miR-10b expression in gliomas with upregulation of RHOC and the urokinase receptor uPAR was observed (Veeravalli *et al*., 2010). These targets were shown independently to directly promote glioma cell invasion and migration (Veeravalli *et al*., 2010). Other oncomiRs targeted the cell cycle and cell survival pathways in GBM include miR221/222 which has been reported to promote oncogenesis *in vitro* and *in vivo* by regulating the STAT3/Akt pathway as well as by direct post-transcriptional regulation of tumor suppressor $p27^{Kip1}$ in glioma cells (Gillies & Lorimer 2007; Zhang *et al*., 2010).

On the other hand, miR-128 is one of the most frequently downregulated miRNAs in gliomas. It has been shown to mainly work through E2F3a and BMI1 pathways, activating cell cycle and increasing cellular proliferation (Godlewski *et al*., 2008).

MiRNAs with tumor suppressor activity in GBM include also miR-7 and miR-34a which also have inhibitory effects on cell cycle and proliferation (Genovese *et al*., 2012; Yin *et al*., 2012). Specifically, ectopic miR-7 expression has been shown to increase cell death in established, primary and tumor-derived GBM stem cell lines in part by targeting EGFR and controlling Akt activity via translational inhibition of upstream Akt activators, IRS1 and 2 (Kefas *et al*., 2008). It was also shown that miR-7 target genes to several different signalling pathways downstream of EGFR and demonstrated that Raf1, an effector of EGFR signalling in the oncogenic Raf-MEK-ERK cascade is also directly regulated by miR-7 at the transcriptional level (Kefas *et al*., 2008; Webster *et al*., 2009). Thus, diminished miR-7 expression in primary GBM is predicted to impact multiple EGFR mediated signalling pathways. The miR-34a locus, which is frequently epigenetically silenced in a spectrum of tumors was identified as a direct p53 transcriptional target and is an important component of the p53 tumor suppressor network (He *et al*., 2007). The highly conserved miR-34a locus, which maps to 1p36, a region frequently lost in gliomas, is expressed at relatively low levels in primary GBM compared to normal brain and has been shown to directly target multiple oncogenes including c-MET, NOTCH 1 and 2 and CDK6 in gliomas (Guessous *et al*., 2010). Consistent with a tumor suppressor function, ectopic miR-34a in a GBM cells suppresses xenograft formation, diminishes G1/S cell cycle progression and promotes cell death (Li *et al*., 2009). Thus, loss of miR-34a expression may promote gliomagenesis by inhibiting p53-mediated apoptosis in primary GBM cells.

To date miR-21 and miR-10b have been also implicated as positive mediators of cell migration and invasion process, while miR-29b, miR-125a and miR-146b have been demonstrated to act as suppressor of GBM invasion and migration. Specifically, RECK (*reversion-inducing-cysteine-rich protein*) and TIMP3 (*metallopeptidase inhibitor 1*), which are inhibitors of matrix metalloproteinases (MMPs) have been shown to be directly targeted by mir-21 to promote GBM cell migration and invasion (Gabriely *et al*., 2008; Conolly *et al*., 2010). The tropomyosin 1, PDCD4, maspin, RhoB and MARCKS loci, which have been shown to be important miR-21 targets in breast and/or prostate cancer cell migration are also likely to play important roles in glioma cell migration.

The additional aspect of gliomagenesis is angiogenesis and tumor metabolism. miR-296 was highlighted as a pro-angiogenic downstream effector of VEGF and PDGFR produced by human endothelial cells (Wurdinger *et al*., 2008).

Regulation of miRNA biogenesis pathway

The expression level of miRNA can be also modified as a result of defects in the miRNAs biogenesis pathway. The deregulation of the miRNA processing has been associated with various cancers and the knockdown of key miRNA biogenesis factors (Drosha, Dicer, exportin-5) enhances tumorigenesis (Sugito *et al*. 2006; Kumar *et al*., 2007; Melo *et al*., 2009; 2010; Faber *et al*., 2011; Ravi *et al*., 2012). The transcriptome and small RNA deep sequencing analysis in glioma showed that altered miRNA-expression profiles is also a result of a widespread gene expression changes, what affects miRNA processing (Moore *et al*., 2013). The maturation process and ob-

served miRNA M/P ratio in gliomas (mature miRNA/pre-miRNA) was modulated by changes in expression of the nuclear processing (SMAD5), cytoplasmic processing genes (DICER 1, EIF2C1, EIF2C2) and the nucleo- cytoplasmic transport (HPO5) as well. These genes can be seen in TGFα/BMP/SMAD signaling pathway and they are involved, through the interaction with helicase p68, in the processing of pri-miR-21 to pre-miR-21 (Davis *et al*., 2009). So, it was evidenced that also the changes within the miRNA biogenesis pathway that impact miRNA maturation are important for gliomagenesis, glioma progression and patient survival (Moore *et al*., 2013).

miRNA-based GBM subtypes profiling-miRNA contribution to GBM subclass phenotype

Extremely unfavourable prognosis of GBM is the main reason to develop more effective diagnostic and therapeutic strategies that are based on a biologically and clinically relevant disease subclassification system.

The recent large-scale multidimensional analyses of molecular characteristics, TCGA, which includes expression profiles of miRNAs along with DNA copy number, gene expression and DNA methylation, have revealed frequent genetic alterations in three critical core pathways (Kim *et al*., 2011). The consensus clustering of GBM samples identified five clinically and genetically distinct subclasses of GBM that are related to a different precursor cell type with robust survival differences. The oligoneural, radial glial, neural, neuromesenchymal and astrocytic classes were identified. These subclasses appeared to predict clinical outcomes more precisely than mRNA profiles and the expression profiles. miRNAs are useful for subclassification of GBM and could identify novel therapeutic targets (Phillips *et al*., 2006; Verhaak *et al*., 2010; Kim *et al*., 2011).

The highly orchestrated and unique progression of miRAs expression accompanies each stage of development. These subtypes, along with the miRNAs expression-based profiling mirror neurogenesis and are distinguishable on the basis of gene expression profiles and specific genomic and signalling alterations. Although many efforts have been made to provide mRNA-based subclassification, there is no meaningful correlation between the GBM subtype and the patients survival or drug resistance, what only miRNA-profiling studies could achieve. Importantly, molecular subtypes of GBM appear to correlate with clinical phenotypes, tumor characteristic and treatment outcomes The identification of multiple subtypes within GBM has underscored the heterogeneity of diseases that all share the same WHO histopathological grade and the need for rigorous molecular classification in order to design appropriate therapeutics and to accurately evaluate therapeutic efficacy in well-defined molecular subsets of GBM. Significant survival benefit of radiation and temozolomide was observed for patients with tumors in the astrocytic subclass, but not for those with tumors in the oligoneural, neural or neuromesenchymal subclasses.

It was also found that miR-9 downregulates the JAK/STAT pathway and serves as a switch that regulates oligoneural *versus* mesenchymal decision in GBM. miR-124 was therefore established to be important in promoting neuronal differentiation and decreasing growth in GBM (Kim *et al*., 2011). It was also shown that, with 88% of sensitivity and 100% of specificity, the upregulation of miR-92b and miR-9/9* could be used to distinguish primary gliomas from metastases (Nass *et al*., 2009).

This approach has revealed that the glioma transcriptome is highly structured and reflects tumor histology, molecular alterations and clinical outcome as well.

Thus, profiling-based classification may have highest clinical relevance in suggesting different therapeutic strategies and could lead to more personalized approaches to treating groups of GBM patients based on their genomic alterations.

Prognostic value of miRNAs — miRNAs in survival

miRNAs discriminate tumor origins, subtypes, oncogenic mutations, cancer predisposition and regulating the most important cellular processes. They are also able to predict cancer prognosis and/or response to specific therapies. It has been recently demonstrated that 10-miRNA expression signature can be an independent predictor of survival of GBM patients (Srinivasan *et al*., 2011). The estimation of the benefit of various cancer therapies to patients is very important and could give the foundation of personalized cancer therapy. While the clinical features like age and Karnofsky performance status are known prognostic markers among GBM patients, MGMT gene promoter methylation status is of great interest in recent times because it predicted response of GBM patients receiving temozolomide chemotherapy in addition to irradiation (Stupp *et al*., 2009).

Several other molecular markers with prognostic and predictive significance in GBMs have been identified (Palanichamy *et al*., 2006). Except for a few recent reports on the role of miRNAs in GBM prognosis, the possibility of prognostic miRNA signatures have not been extensively investigated (Zhi *et al*., 2010).

The ten miRNA signature included three miRNAs (miR-20a, miR-106a and miR-17-5p) that were protective and seven miRNAs (miR-31, miR-222, miR-148a, miR-221, miR-146b, miR-200b and miR-193a) that were risky with respect to their association between their expression and patient survival were described (Srinivasan *et al*., 2011) (Table 1). The protective miRNAs were expressed at a higher level in the low risk compared to the high risk group. On the other hand, the expression risky miRNAs was higher in the high risk than in the low risk group. The nature of these miRNAs is suggestive of their functions being either inhibitory or promoting, respectively, of various properties of cancer cells like proliferation, migration and invasion. Expression profiles of miRNA are useful for predicting GBM patient survival and have the potential to identify efficacious therapeutic targets. Up to now, there is one clinical trial with miRNA (http://clinicaltrials.gov). This study tests the hypothesis that in primary glioma samples miR-10b expression patterns will serve as a prognostic and diagnostic marker.

The role of miRNA in response to therapy

Drug resistance

Chemotherapy is the treatment of cancer with single or multiple cytotoxic drug which mostly work by inhibiting the proliferation of actively dividing cells. These drugs include alkylating agents, platinum agents, nitrogen mustards, antimetabolites, anthracyclins, alkaloids or taxanes (Malhotra & Perry, 2003). Nonspecific cell targeting and late stage side effects of chemotherapy has led the way towards designing targeted therapy agents which specifically target the cancer cells by blocking the function of dysregulated proteins in oncogenic pathways. Small molecule inhibitors (mostly tyrosine kinase inhibi-

Table 1. The list of potential diagnostic and prognostic miRNAs identified in GBM (referenced in the text).

Diagnostic and prognostic miRNA in GBM				
Survival	Response to therapy		Circulating miRNA	Cancer stem cells (CSC)
	Drug resistance	Radioresistance		
miR-20a miR-106a miR-17-5p miR-31 miR-222 miR-221 miR-148a miR-146b miR-200b miR-193a	**TMZ resistance** miR-21 miR-125b-2, miR-95, miR-455-3p, miR-10a, miR-181d **TRAIL resistance** miR-21 miR-30b miR-30c	let-7 family miR221/222 miR-425 miR-93	miR-15b miR-17-5p miR-20a miR-23a miR-31 miR-106a miR-146b miR-148a miR-150 miR-193a miR-197 miR-200b miR-221 miR-222 miR-548-5p	miR-128 miR-137 miR-34a miR-326

tors TKIs) and monoclonal antibodies are two major classes of targeted therapy agents (Gerber, 2008).

However recently, miRNA have been associated with drug resistance to both chemo- and targeted therapies (Table 1). miRNA studies have revealed that aberrant miRNA expression could affect chemosensitivity and have also identified several miRNAs associated with TMZ resistance: miR-21, miR-125b-2, miR-95, miR-455-3p, miR-10a, miR-181d (Shi *et al*., 2010; 2012; Ujifuku *et al*., 2010). It was shown also that the overexpression of miR-21 and miR-145 make the cancer cells resistant to sunitinib and temozolomide (Costa *et al*., 2013) (Table 1).

As well as affecting TMZ, miR-21 along with miR-30b and miR-30c have been identified as regulators of TNF-related apoptosis-inducing ligand (TRAIL). These three miRNAs therefore could affect the sensitivity of glioma cells to treatment with the TRAIL ligand, since theirs upregulation was shown in TRAIL-resistant glioma cell lines (Quintavalle *et al*., 2013) (Table 1).

miRNA associated with radioresistance

Another effective cytotoxic therapy for GBM patients is radiotherapy. Several reports revealed that miRNAs dysregulation also affect the radiosensitivity of glioma cells, e.g. let-7 family, miR-221/222, mir-425 and miR-93 (Li *et al*., 2011; Chen *et al*., 2012; Gwak *et al*., 2012). miR-21, commonly upregulated in GBM, also play a crucial role for radiosensizitation of GBM by modulating a tumor suppressor network and phosphoinositide kinase (PI3K)/AKT pathway (Papagiannakopoulus *et al*., 2008; Gwak *et al*., 2012) (Table 1).

miRNA and cancer stem cells

The cancer stem cells (CSC) hypothesis is also very interesting in terms of the miRNA function. In the light of that tumors are driven by a small subpopulation of cells with stem cell-like properties. This may provide novel insights into the radio- and chemoresistance of GBM (Magee *et al*., 2012).

Some miRNAs have been identified to contribute to CSC properties and cancer heterogeneity, like: miR-128, miR-137, miR-34a, miR-326 (Godlewski *et al*., 2008; Silber *et al*., 2008; Kefas *et al*., 2009; Li *et al*., 2009; Guessos *et al*., 2010) (Table 1).

miRNA in biofluids — circulating miRNAs

Histopathology of tumor specimens gained by microsurgical resection or by stereotactic biopsy is still the standard diagnostic procedure for patients with glioma. Neuroimaging, in particular MRI, is then instrumental for disease staging and for follow-up treatment. To date, there are no biomarkers in blood and serum or cerebrospinal fluid (CSF) for detection, follow-up, or prognostication of gliomas. To prevent degradation in the circulation, miRNAs are released by cells in both exosomes (lipid vesicles) and miRNA/protein complexes (Yang *et al*., 2012). miRNA signatures have been identified in both GBM tissue and circulation: plasma and cerebrospinal fluid (CSF) of glioblastoma patients (Duffy *et al*., 2011; Hua *et al*., 2012). 33 up-regulated and 40 down-regulated miRNAs have been found. Based on research of various biofluids, a panel of 15 candidate miRNAs biomarkers has been constructed (Tumilson *et al*., 2014). Some of them include miRNAs highlighted as linked to gliomagenesis and function, including: miR-17-5p, 21, 15b, 221 or 222 or to the chemo- and radiotherapy: miR-21, miR-15b, miR-181, miR-30b, c and miR-93 (Tumilson *et al*., 2014) (Table 2). Although, circulating miRNA are ideal candidates for diagnostic and prognostic indicators, there is still need to overcome some general obstacles, e.g. standardization of isolation and analysis techniques to improve the reliability of miRNAs biomarker data. Because of introduction of molecular targeted drugs and individualized therapy for GBM treatment, identification of meaningful biomarkers is crucial for therapeutic strategy and predicting tumor recurrence (Sathornsumetee *et al*., 2007; Huang *et al*., 2009; Polivka *et al*., 2009; Thaker *et al*., 2009). Moreover, a combination of biomarker genes has been reported to be more useful than a single one, resulting in a multitarget strategy treatment (Colman *et al*., 2010). Present data already show, that miRNA are involved in many cellular processes that are altered in GBM tumors, such as angiogenesis, invasion, cell proliferation and apoptosis. miRNAs due to their multifunctionality and highly controlled expression level, could be a good potential diagnostic and prognostic biomarkers for GBM. The list of described above miRNAs is given in the Table 1.

THERAPEUTIC STRATEGIES FOR TARGETING miRNAs

miRNA targeting tools against glioma

The discovery that miRNAs can function as oncogenes or tumor suppressors shed light on the possibility of using these molecules for GBM therapeutic intervention. This new modality of targeted molecular intervention is promising for the development of an optimal, reliable, less toxic and effective personalized treatment for glioblastoma. However, modulating a single microRNA can affect many different pathways, since, as opposed to shRNA (small hairpin RNA), one miRNA can target dis-

Table 2. Direct-miRNA-based therapeutic approaches (according to the data from Garzon *et al.*, 2010).

miRNA-based therapeutic approaches	
Inhibition of oncomiRs (antagomiRs)	Replacement of tumor-supressive miRNA
• 2-O-Me PS oligonucleotides (2′O –methyl with phosphorothioate modifications)) • 2-O-Me PS oligonucleotides with cholesterol backbone • LNA (locked nucleic acid) • miRNA sponges • miR-masks • small-molecule inhibitors	**Non-viral based approach** • miRNA mimic agents **Viral-based approach** • adenovirus associated vectors (AAVs)

tinct molecules that are involved in different oncogenic pathways (Garzon *et al.*, 2010).

To date, there are two main strategies to modulate microRNA expression in cancer: silencing an oncogenicmiRNA or overexpressing a previously downregulated tumor suppressor miRNA (Table 2). Oncogenic microRNAs can be downregulated by: (a) using antisense miRNA nucleotides (AMOs or antagomirs), (b) direct targeting by miRNA sponges, (c) indirect targeting by pharmacological agents, and (d) miRNA masking.

AMOs are synthetic oligonucleotides that present a complementary sequence to the mature targeted miRNA. They mainly work by competitively blocking the interaction between the miRNA and its aimed mRNA. As already shown by several *in vivo* studies, this technique presents a high miRNA-silencing efficacy (Elmen *et al.*, 2008). In the murine brain, a complete miRNA-21 eradication and increased glioma apoptosis was achieved by treating glioma cells with LNA-antimir-21 in the presence of neural precursor cells expressing S- TRAIL (variant of tumor necrosis factor-related apoptosis inducing ligand) (Corsten *et al.*, 2007). However, since AMOs activity is sequence-specific but not gene specific, it can elicit unwanted off-target side effects and toxicity. Therefore, to achieve high specificity, binding affinity and potency, an AMO might need specific chemical modifications or an optimization of its structure. Based on previous exciting results, one clinical trial has been started in Denmark, using LNA-anti-microRNA-122 in human subjects (http://clinicaltrials.gov). MiRNA sponges are competitive miRNA inhibitors that contain multiple binding sites for an endogenous microRNA, attaching to the miRNA of interest and capturing it into a nonfunctional complex. Since sponges are designed with a complementary heptameric seed, a single sponge can be used to repress an entire miRNA family (Ebert *et al.*, 2010; Gumireddy *et al.*, 2008). Although they seem to be as effective as AMOs, further studies evaluating their efficacy *in vivo* are still needed. On the other hand, drugs, such as chemotherapy agents or small molecule targeted inhibitors, can also downregulate an oncogenic miRNA by regulating a transcriptional factor that later on will modulate miRNA expression (Gumireddy *et al.*, 2008). This link is considered of high affinity and stability, decreasing potential side effects of mRNA degradation (Xiao *et al.*, 2007).

Alternatively, a previously downregulated tumor suppressor miRNA can be amplified by: (a) synthetic oligonucleotides mimicking mature miRNA, (b) pharmacological agents, and (c) adenovirus-associated vectors (AAVs). While very effective and stable in *in vitro* studies, synthetic oligonucleotides can be poorly stable and present delivery issues in *in vivo* models (Garzon *et al.*, 2010). To overcome this issue and achieve a better therapeutic effect, polymer and lipid-based nanoparticles have been developed for systemic delivery in *in vivo* models (Merritt *et al.*, 2008). However, further studies are still necessary for translating this therapeutic approach into clinical practice. Primarily, to assure a proper drug delivery, the instability of unmodified oligonucleotides in biological fluids needs to be excelled, so the compound would not be rapidly degraded. Moreover, off-target effects and safety issues still need to be overcome.

Non-immune off-target effects, such as unwanted gene silencing in non-target tissues leading to phenotypic changes, are one of the major obstacles for a clinical application of miRNA- based therapies. This issue mainly occurs due to miRNA cross-hybridization with genes that present partial complementarity. Systemic overexpression of targeted miRNA using a synthetic mimic could target genes in particular in non-cancerous tissues (e.g. bone development, immune function (Th1 responses) and granulocytic differentiation), and cause unwanted side effects such as autoimmunity or hyperproliferation. These problems could be solved by engineering effective systems that deliver the synthetic miRNA oligonucleotides specifically to the diseased tissue and cancer cells. Vector sequence optimization, such as 2'-O-methylation, is presented as an inviting solution (Jackson *et al.*, 2006).

AAV is a known used method of delivering miRNAs that has been tested in clinical trials (Michelfelder & Trepel, 2009). This is also a relatively safe delivery method, since AAVs do not integrate into the host genome and can be efficiently eliminated, minimizing the risk of vector-related toxicities. Such findings support the idea that the selection of miRNAs that are overexpressed in normal tissues, but downregulated in tumors, can be an effective and minimally toxic way for restoring missing tumor suppressor microRNAs in anticancer therapy (Michelfelder & Trepel, 2009).

Furthermore, miRNA masking has arisen as a promise to decrease AMOs off-target effects. MiR-mask is a designed sequence that perfectly attaches to the microRNA endogenous binding site in the aimed gene. Subsequently, the target gene forms a highly stable complex with the mRNA, blocking miRNA access to the gene-binding site.

Delivery issue

Many obstacles must still be overcome to establish miRNA-targeted therapies. One of the most critical issues is how to deliver the agent (an miRNA mimic or inhibitor) to brain protected by the blood–brain barrier (BBB).

Delivery of miRNA-based vectors to brain tumors is particularly challenging due to the other biological hurdles, such as intravascular degradation, reticuloendothelial system trapping and tissue penetrance. To date, some specially modified nanoparticles and payload-conjugated peptide (paclitaxel derivative) ANG1005 was shown to penetrate the BBB (Thomas *et al.*, 2009). However, despite few successful attempts, it is still unlikely that a

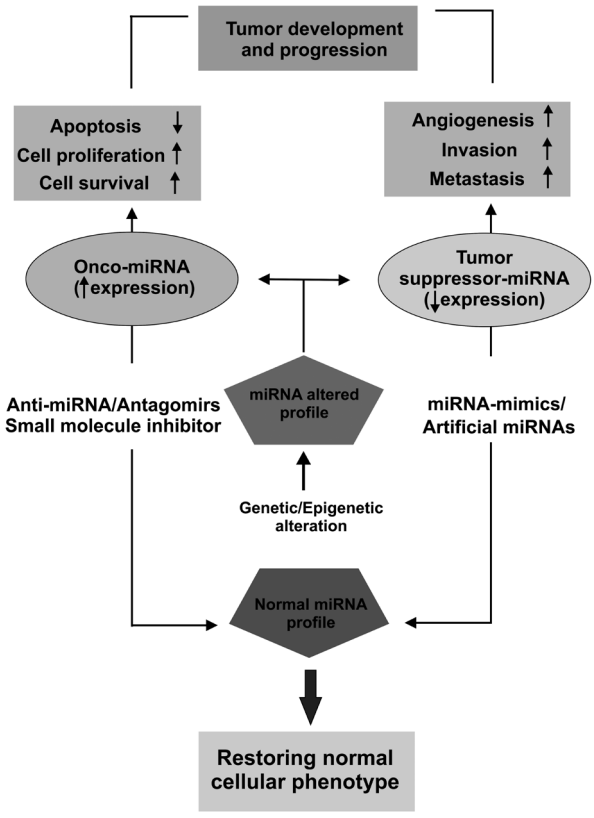

Figure 3. Schematic overview of miRNAs-based treatment strategies.
miRNAs modulate multiple mechanisms leading to cancer initiation, progression and dissemination. The cancer treatment strategies can involve antagomiRs and miRNA-mimics to restore the normal phenotype.

systemically administered vector could deliver its payload in an optimal and dose-effective concentration (Black & Ningaray, 2004); Liu *et al*., 2010).

Local delivery techniques could potentially improve the efficacy for GBM, since systematic metastasis is extremely rare in GBM compared with other types of tumor. Convection- enhanced delivery (CED) is one of the most promising local delivery techniques to improve the efficacy of recent treatments, including conventional chemotherapy, molecular-targeted therapy, and gene therapy (Zhou *et al*., 2012). Interesting local delivery options are also viruses encoding miRNAs/inhibitors (lentivirus), AAVs and liposomal nanoparticles. As oppose to a brief therapeutic effect usually offered by nanoparticles, virus modulation presents a long and high expression. Due to serum nucleases and rapid renal clearance, naked vectors are frequently unstable in the circulation. To solve this issue, chemical modifications of oligonucleotides and nanoparticle encapsulation are presented as good options also as an improvement of CED for the treatment of brain tumors (Hadjipanayis *et al*., 2010). Moreover, to avoid and overcome clearance by the reticuloendothelial system, nanoparticles with less than 100 nm in diameter should be preferentially used (Bumcrot *et al*., 2006).

Novel and very interesting targets for miRNA-based therapy are tumor-derived exosomes and secreted miRNA. Recent investigations have revealed that exosomes secreted by tumor cells contain numerous functional miRNAs, which could play important roles in tumor initiation and progression (Valadi *et al*., 2007). Secreted miRNAs derived from tumor cells may play important roles in intercellular communication, since miRNA transferred from tumor cells could regulate protein expression in the surrounding structure (Katakowski *et al*., 2010). This recent investigation into the biological function of exosomes suggests the opportunity to create a new miRNA delivery system and this intercellular mechanism could represent a novel therapeutic target for the future (Alvarez-Erviti *et al*., 2011; Mizoguchi *et al*., 2013).

CONCLUSION AND PERSPECTIVES

MicroRNAs have been suggested by a large number of studies to play a pivotal role in the development of malignant phenotype of glioma, characterized by enhanced cell survival, proliferation, tumor angiogenesis, differentiation as well as generation of cell stemness.

These findings evolved into the characteristic as being the useful potential GBM biomarkers. Discoveries of the biological significance of miRNAs dysregulation in glioma cells not only fill some gaps between previously identified, but disconnected, mechanistic components underlying the pathogenesis of the disease, but also provide a model system through which the role of miRNA in tumorigenesis and cancer progression can be better understood. Moreover, it has been increasingly revealed that changes in the expression level of particular miRNAs might represent a new class of benchmarks indicative of the presence and/or progression of glioma tumors, and therefore might be of diagnostic or prognostic value, thus fully complement the idea "from the bench

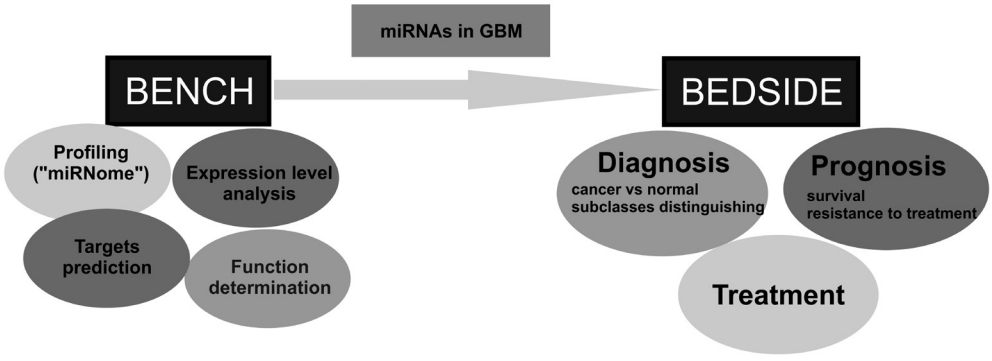

Figure 4. The multifunctionality of miRNAs — from the bench to bedside.
The expression-based profiling of miRNAs gives the basis to the diagnosis (subclassification among the samples that all share the same WHO histopathological grade) and prognosis (survival, chemo- and radiotherapy resistance) of the tumor. The highly deregulated in GBM miRNAs could be the target for effective molecular treatment.

to bedside" (Fig. 4). Furthermore, it is important also to acknowledge that as one of the key molecules involved in mediating cellular behaviors essential for establishment of primary tumors as well as invasive growth of glioma, miRNAs are potentially promising targets of future anti-glioma intervention, despite the apparently pressing challenges lying ahead along the path towards the eventual clinical application of miRNA-based therapies (Fig. 4). Thus, further and more in-depth mechanistic studies on the biological basis upon which miRNAs contribute to gliomagenesis and lethality of the disease, as well as translational research to overcome the technical barriers impeding the applicability of miRNA as diagnostic, prognostic, therapeutic or preventive tool, are equally urgent. Although promising, the above findings raise a very important question about how can this knowledge be translated into clinical practice. It seems that the most rising challenge is to develop a reliable, commercially available and non-expensive assay to detect miRNA from patients sample and then the miRNA expression assays should be added to wide clinical trials. Thus, a more personal treatment could be offered for each patient condition, and depending on the miRNA profile, the right treatment match and follow up could be chosen for each patient. Based on this idea, also specific chemotherapy or radiotherapy regimens could be selected depending on distinct miRNA expression profiles.

A useful future application of miRNA-based therapies could be the local delivery use in combination with standard therapeutic approaches, such as surgery and chemotherapy, which will certainly increase cell-specific target delivery and reduce normal cell direct toxicity.

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Conflicts of interests

The author declares no conflicts of interests.

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